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Contact lens care solution killing efficacy against *Acanthamoeba castellanii* by *in vitro* testing and live-imaging

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ABSTRACT

In the past decade there has been an increased incidence of *Acanthamoeba* keratitis, particularly in contact lens wearers. The aim of this study was to utilize in vitro killing assays and to establish a novel, time-lapse, live-cell imaging methodology to demonstrate the efficacy of contact lens care solutions in eradicating *Acanthamoeba castellanii* (*A. castellanii*) trophozoites and cysts. Standard qualitative and quantitative in vitro assays were performed along with novel time-lapse imaging coupled with fluorescent dye staining that signals cell death. Quantitative data obtained demonstrated that 3% non-ophthalmic hydrogen peroxide demonstrated the highest percent killing at 87.4% corresponding to a 4.4 log kill. The other contact lens care solutions which showed a 72.9 to 29.2% killing which was consistent with 4.3–2.8 log reduction in trophozoite viability. Both analytical approaches revealed that polyquaternium/PHMB-based was the least efficacious in terms of trophicidal activity. The cysticidal activity of the solutions was much less than activity against trophozoites and frequently was not detected. Live-imaging provided a novel visual endpoint for characterizing the trophocidal activity of the care solutions. All solutions caused rapid rounding or pseudocyst formation of the trophozoites, reduced motility and the appearance of different morphotypes. Polyquaternium/alexidine-based and peroxide-based lens care system induced the most visible damage indicated by significant accumulation of debris from ruptured cells. Polyquaternium/PHMB-based was the least effective showing rounding of the cells but minimal death. These observations are in keeping with care solution biocides having prominent activity at the plasma membrane of *Acanthamoeba*.

1. Introduction

Acanthamoeba is a microscopic, free-living amoeba found worldwide and typically in low tonicity, water and soil [1]. It exists as a feeding/replicating trophozoite stage that, in response to adverse conditions, can transform into intermediate variants that in due course will result in resistant double-walled cysts [2,3]. *Acanthamoeba* keratitis (AK) is a serious infection that can result in permanent visual impairment or blindness. Several factors are involved in increasing its visual morbidity including the aggressive nature of the parasite, difficulties in diagnosis and reduced immune status of the host [4–7]. Due to the overall difficulty in diagnosing and treating AK, much effort of late has focused on prevention.

In the US, an estimated 85% of cases of AK occur in soft contact lens wearers. Commonly cited data for the incidence of AK in developed countries is 1.65–2.01 cases per million contact lens wearers, although more recent studies report 17–70 per million [8–13]. However, the incidence has been shown to vary under different conditions including seasonal changes, regional flooding and alterations in contact lens care [10,12,14–15]. In 2007 an outbreak of AK prompted a voluntary recall of a contact lens care system (AMO Complete Moisture Plus) [10]. A multivariate risk factor analysis demonstrated a 17-fold risk of AK when using this particular solution [12]. Risk factors for contact-lens associated AK include: improper lens storage, improper handling or disinfection of lenses, wearing lenses while swimming, showering or using hot tubs, and a history of corneal trauma [10]. Recent studies have focused on patient influenced compliance factors such as topping off and reuse of contact lens care solutions [12,16–18]. An increased incidence of AK has also been related to an Environmental

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Protection Agency-mandated reduction of chlorine levels in municipal water supplies affecting both tap water and recreational water [19]. Several studies have addressed the efficacy of various contact lens care solutions against *Acanthamoeba* although there is no standardized method for this [20–25]. Also there is no requirement for testing against *Acanthamoeba* for a contact lens care product to be compliant with international standard ISO 14729 for marketing purposes [26]. Of the available contact lens care solutions hydrogen peroxide based products are the most efficacious against both *Acanthamoeba* trophozoites and cysts [26]. The aim of this study was to utilize qualitative and quantitative *in vitro* killing assays and to establish a novel, time-lapse, live-cell imaging methodology to demonstrate the efficacy of contact lens care solutions in eradicating *Acanthamoeba castellanii* (*A. castellanii*) trophozoites and cysts.

2. Materials and methods

2.1. Acanthamoeba *culture procedures*

A. castellanii strain 50370 (American Type Culture Collection, Manassas, VA), a strain capable of producing contact lens related keratitis as established by Buck et al. [27], was used for these experiments. Trophozoites were grown in tissue culture flasks in axenic potato–glucose–yeast 712 (PGY-712) medium (Difco, Detroit, MI) at 30 °C. Cysts were prepared from late-log-phase trophozoite cultures using Neff's Encystment medium as previously established by Hughes et al. [28]. Briefly, the trophozoites were washed three times in encystment medium by centrifugation at $1000 \times g$ for 10 min. An aliquot of the trophozoites was counted and 10^7 trophozoites were added into 100 ml of encystment medium in a T-175-cm^2 tissue culture flask with a filter cap. The cultures were incubated at 30 °C for 7–10 days. The cultures were examined daily and harvested at the end of 7–10 days when microscopic examination showed that 95% of the cells were mature cysts. The cysts were harvested from the flask by gently scraping with a rubber policeman. The cysts were centrifuged at $1000 \times g$ for 10 min. The pellet was washed in 1/4 strength Ringer's solution by centrifugation and used within 4 h post-harvesting. Cyst and trophozoite populations were counted using a hemocytometer. Each preparation was counted 3 times and the average number of these readings was used to determine the number of cells per ml. Stock solutions of 1×10^5 trophozoites or cysts/ml were prepared for qualitative experiments and stock solutions of 5×10^5 cells/ml were used for quantitative experiments [26,27].

2.2. Preparation of Escherichia coli *bacterial lawns*

E. coli (strain 8739, American Type Culture Collection, Manassas, VA) was initially cultured in Nutrient broth (Difco, Detroit, MI) and streaked onto an agar plate. A single colony was selected and incubated at 37 °C with shaking at 250 rpm overnight. The culture was expanded in 50 ml medium for 2 h before centrifuging at $1300 \times g$ for 20 min. The medium was decanted and the bacterial pellet resuspended in 1/4 strength Ringer's solution. The optical density of the pellet was adjusted to 0.4 at 600 nm [29]. *E. coli* so processed was used to prepare bacterial lawns on 1.6% non-nutrient agar (Becton, Dickinson and Company, Sparks, MD) plates to supply nutrient to the trophozoites and cysts through the incubation period of the qualitative experiments. The *E. coli* were plated onto 4 ml of solidified non-nutrient agar in a flat-bottom six well plate. The plates were gently rotated via a circular motion to ensure even spread of the bacterial suspension. The plates were placed for 1 h at room temperature in a biosafety cabinet to allow formation of a smooth dense bacterial lawn of *E. coli*.

2.3. Quantitative Acanthamoeba *assays*

Five commercially available contact lens cleaning and disinfecting solutions were tested: polyquaternium/PHMB-based (Bausch + Lomb, Rochester, NY), peroxide-based lens care system (Alcon, Fort Worth, TX), myristamidopropyl dimethylamine/polyquaternium-1 (Alcon, Forth Worth, TX), Polyquad/aldox-based multipurpose solution (Alcon), and polyquaternium/alexidine-based (AMO, Santa Ana, CA). Peroxide-based lens care system was utilized without the neutralizing disk. All solutions were tested from previously unopened, unexpired bottles. Table 1 lists the biocides present in each solution. Non-ophthalmic 3% hydrogen-peroxide (Sigma, St. Louis, MO) and growth medium controls (PGY 712 medium for trophozoites and Neff's medium for cysts) were also studied. Quantitative experiments were performed using microtiter plate assays to determine the minimum amoebicidal activity of the test and control solutions against *A. castellanii* trophozoites and cysts [30]. Briefly, 5 ml of test or control solutions were challenged with 5×10^4 trophozoites or cysts. 100 μl aliquots from each inoculated test solution were removed and plated (ten-fold serial dilutions) into 96 well microtiter plates in quadruplicate at 0, 1, 2, 4, 6 and 24 h. The initial dilution was made in Tween 80-lecithin neutralizing medium and subsequent serial dilutions were made in 1/4 0/0 Ringer's solution. 10 μl of a suspension of *E. coli* (approximately 5×10^7 bacteria/ ml) were added into the wells and the plate sealed and incubated at 30 °C for 14 days. The plates were visualized every other day to determine the growth of *A. castellanii* and the number of wells positive for amoebic growth was enumerated at the end of 14 days. The number of surviving trophozoites/cysts was estimated using an established most-probable number calculation based on the Spearman–Karber computations using the presence or absence of excystment in the wells and taking into account the 10 fold serial dilutions [30–31]. The percent surviving organisms, from where the percent killing was calculated, was based on the Karber number obtained versus the number of surviving organisms at the "0" time point.

Table 1
Biocides in each of the care solutions tested.

Solution	Biocide
Peroxide-based lens care system (CLEAR CARE®)	3% hydrogen peroxide
Polyquaternium/PHMB-based (Biotrue™)	Polyaminopropyl biguanide (0.00013%) Polyquaternium (0.0001%)
Myristamidopropyl dimethylamine/polyquaternium-1 (OPTI-FREE® Pure Moist®)	Myristamidopropyl dimethylamine (0.0006%) Polyquaternium-1 (0.001%)
Polyquad/aldox-based multipurpose solution (OPTI-FREE® RepleniSH®)	Myristamidopropyl dimethylamine (0.0005%) Polyquaternium-1 (0.001%)
Polyquaternium/alexidine-based (RevitaLens OcuTec®)	Alexidine dihydrochloride (0.00016%) Polyquaternium-1 (0.0003%)

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